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ABSTRACT

Retinoids are vitamin A derivatives, which cause growth inhibition, differentiation and/or apoptosis in various cell types, including some breast cancer cells. In general, estrogen receptor (ER)-positive cells are retinoic acid (RA) sensitive, whereas ER-negative cells are resistant. I have showed that ER-negative MDA-MB-231 cells and MDA-MB-468 cells are strongly growth inhibited by retinoids in combination with PKC inhibition, and inhibition of PKCδ in particular. In this report, I show that the PKC inhibitor GF109203X increases gene regulation by RA, as shown by microarray studies, and identify certain genes/pathways that may be important. I also show that apoptosis following treatment with RA plus the PKCδ specific inhibitor Rottlerin involves loss of mitochondrial transmembrane potential and release of cytochrome c into the cytosol. Further, I report that while GF109203X decreases phosphorylation of RARα in MDA-MB-231 cells, stable expression of ERα or β in these cells leads to an increase in RARα phosphorylation.

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Introduction

Retinoids are derivatives of vitamin A, which induce differentiation and growth inhibition in a variety of cell types, including breast cancer cells [1-3]. They act mainly by binding to nuclear retinoid receptors, RARs and RXRs, which act as ligand regulated transcription factors [4]. Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats [5-7], and there is some clinical evidence that retinoids may be beneficial in breast cancer prevention [8, 9]}. Furthermore, it has recently been proposed that a synthetic "rexinoid" (RXR selective retinoid) may prevent the development of multidrug resistance following exposure to chemotherapy agents such as Taxol [10-12]. Thus, further studies of the potential use of retinoids in therapy of breast cancer are warranted and for this purpose it is important to clarify how retinoids exert their effects on breast cancer cells and what determines sensitivity vs. resistance to these compounds.

Inhibition of growth by retinoic acid (RA) in human breast cancer cells *in vitro* generally correlates with expression of the estrogen receptor (ER) α. Most ERα-positive cell lines are growth inhibited in response to retinoids, whereas ERα-negative cells are resistant [13, 14]. We have previously shown that ERα modulates the transcriptional activity of the retinoic acid receptor (RAR) in a ligand independent manner, via its N-terminal AF-1 domain [15]. In addition, we have reported that stable overexpression of ERβ also restores sensitivity to RA in MDA-MB-231 cells [16].

There is also considerable evidence of crosstalk between retinoid signaling and signal transduction pathways activated by growth factors as well as stress stimuli. For example, there have been several reports of crosstalk, both positive and negative, between retinoids and PKC [17-20] as well as well as between retinoid signaling and the MAPK pathways [21-27]. In relation to this, we have reported that several PKC inhibitors strongly potentiate the response of MDA-MB-231 to RA, and that this involves regulation of PKCδ in particular, as well as *activation* of ERK [28].

The approved Statement Of Work for this award proposes experiments described in two main tasks: **1) Define the signal transduction pathways whereby inhibitors of PKC restore sensitivity to retinoids in ER-negative cells**, and **2) Test the hypothesis that expression of ER alters the phosphorylation status of RAR and/or RXR, thereby rendering the cells sensitive to RA.** In this annual report, I will discuss studies undertaken to follow up my 2004 publication, aimed at further elucidating the mechanisms whereby PKC inhibitors synergize with retinoids to induce apoptosis in ER-negative cells (Part 1). I will also describe studies of the phosphorylation status of RARα (Part 2).

Body/Results

1a) Cotreatment with GF109203X increases regulation of gene expression by RA.

I previously showed that the PKC inhibitor GF109203X (GF) enhances RAR mediated transcription from a transiently expressed promoter (Oncogene 2004). To examine the effect of GF on regulation of endogenous gene expression by RA, I chose microarray analysis, which was performed in collaboration with Ligand Pharmaceuticals, Inc. (San Diego, CA). RNA was isolated from MDA-MB-231 cells treated for 24 hours with vehicle, RA, GF, RA+GF, Targretin (bexarotene) or Targretin+GF. Statistical analysis of the resulting data showed that the combination of RA+GF caused a significant change in the expression of many more genes than either RA or GF alone (**Figure 1**). Of note, the rexinoid Targretin (T) changed the expression of

many more genes than RA, however T+GF was no more effective than T alone. This is consistent with the finding that GF did not enhance RXR mediated transcription [28].

Initial studies of the data also identified some potentially interesting genes and pathways that are regulated by RA and/or RA+GF. First, several previously known RA target genes (including RIP140, IL1-β and SCAP2/RA70 [29],[30],[31]) were weakly induced by RA alone but much more strongly by RA+GF (**Figure 2**), again confirming that GF enhances transcriptional regulation of at least some RA targets in these RA resistant cells. Second, the TGFβ pathway may be involved in the effects of RA+GF on cell growth, since several genes in this pathway appear to be regulated by RA and/or RA+GF. Third, some specific genes related to the induction of apoptosis and/or cellular stress were induced by RA+GF. These include a Bcl-2 associated transcription factor, as well as three heat shock proteins, which may suggest an apoptotic response that involves endoplasmic reticulum (ER) stress [32].

1b) Induction of apoptosis by RA + Rottlerin (PKCδ inhibitor) involves loss of mitochondrial transmembrane potential and release of cytochrome c.

Inhibition of PKCδ by Rottlerin or siRNA leads to increased sensitivity to RA in ER-negative MDA-MB-231 and MDA-MB-468 cells ([28] and **Figure 3**). I have begun to investigate the mechanism whereby apoptosis is induced, and discriminate between activation of the intrinsic *vs.* the extrinsic apoptotic pathway. As shown for RA+GF [28], RA+Rottlerin causes cleavage of PARP (**Figure 3C**), indicating activation of downstream effector caspases. To examine more upstream effects, mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed using the fluorescent dye JC-1 **(ref)** and flow cytometric analysis. This showed some loss of $\Delta\Psi_m$ in cells treated with Rottlerin alone, but this effect was more evident in cells treated with RA+Rottlerin (**Figure 4A**). Loss of $\Delta\Psi_m$ was also indicated by the release of cytochrome c into the cytosol (**Figure 4B**). This was shown by Western analysis using S100 fractions, representing cytosolic proteins [33]. Interestingly, we also observed an increase in *total* levels of the proapoptotic protein AIF (data not shown).

Loss of $\Delta\Psi_m$ and the subsequent release of cytochrome c and other proapoptotic factors are hallmarks of the intrinsic apoptotic pathway, since cytochrome c is required for assembly of the apoptosome which activates caspase 9. However, apoptosis initiated and regulated at the level of death receptors (extrinsic pathway) as well as the ER, Golgi apparatus and/or lysosomes ultimately lead to loss of $\Delta\Psi_m$ [34], so no definitive conclusion can yet be drawn from these results.

2a) GF109203X reduces basal phosphorylation of RARα

It has been reported that phosphorylation of RARα by PKC reduces RA induced transcription [17]. Thus, I had postulated that inhibiting PKC in RA resistant breast cancer cells (which display elevated PKC activity) may increase RA induced transcription, leading to improved RA response. To test if GF109203X, which indeed increases RA response in these cells, alters the phosphorylation status of RARα, I performed *in vivo* labeling experiments with transiently expressed Flag-RARα. The cells were incubated with ^{32}P-orthophosphate, cell lysates were prepared and Flag-RARα was immunoprecipitated using anti-Flag resin (Sigma). Precipitated proteins were separated by PAGE and visualized by autoradiography. Several kinase inhibitors were tested for their ability to suppress phosphorylation of RARα. As expected, inhibition of PKC with GF109203X strongly suppressed phosphorylation of Flag-RARα (**Figure 5**). Inhibition of MEK/ERK or JNK also seemed to suppress phosphorylation, but less strongly, and inhibition of p38MAPK had no effect.

2b) Stable expression of ERα or β increases basal phosphorylation of RARα.

Since the ER positive and RA sensitive cell line MCF-7 displays less basal phosphorylation of RARα, and also lower PKC activity ([35] and our observations) than MDA-MB-231, I wanted to assess RARα phosphorylation in MDA-MB-231 stably expressing ER. I first used the S30 cell line, which stably expresses ERα [36], and in contrast to the hypothesis, found that these cells displayed *increased* basal phosphorylation of RARα (**Figure 6A**) and also slightly enhanced PKC and MAPK activities (not shown). I also tested MDA-MB-231 stably expressing ERβ [16] and found similar results, with RARα phosphorylation and kinase activities either increased or unaltered (**Figure 6B** and data not shown). Thus, the increased RA-responsiveness seen in these cells is likely different from the effect observed with the PKC inhibitors (above).

Deviations from the approved Statement Of Work

* Task 1b: Cell lines stably overexpressing PKC (wt or dominant negative) have not been utilized, since downregulation of PKC α and δ using siRNA proved to be a better method.
* Task 1d: Expression of RAR target genes in response to RA +/- GF was assessed by microarray analysis.

List of Key Research Accomplishments

* Confirmed that GF significantly increases regulation of transcription by RA, using microarray analysis, and identified some potentially interesting genes/pathways that may be involved in apoptosis induction by RA+GF.
* Confirmed that inhibition of PKCδ enhances RA-sensitivity in ER-negative MDA-MB-468 cells.
* Showed that induction of apoptosis by RA + the PKCδ inhibitor Rottlerin involves loss of mitochondrial transmembrane potential and release of cytochrome c.
* Showed that GF109203X reduces basal phosphorylation of RARα in MDA-MB-231.
* Showed that stable expression of ERα or β increases, rather than decreases phosphorylation of RARα.

Reportable outcomes

Peer-reviewed articles:

Rousseau, C., Nichol, J, Pettersson, F., Couture, M. C., Miller, W.H, Jr. (2004) *ERbeta sensitizes breast cancer cells to retinoic acid: Evidence of transcriptional cross-talk.* Mol Cancer Res **2**: 523-531

Conclusions

During the second year of this project, the main progress has been in relation to Aim 1, where I have started to elucidate mechanisms whereby PKC-inhibitors may enhance apoptosis in combination with retinoids.

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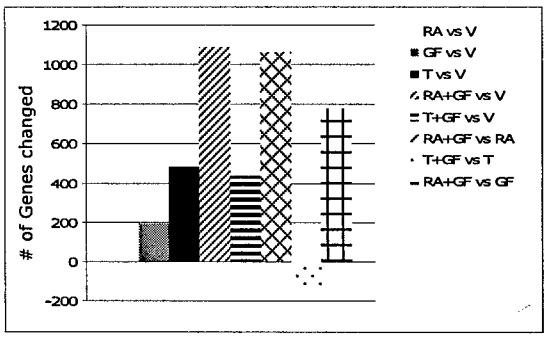
Appendices

1) Figures (2 pages)
2) Article: Rousseau, C, Nichol JN, Pettersson F, Couture MC, Miller WH Jr., *ERbeta sensitizes breast cancer cells to retinoic acid: Evidence of transcriptional cross-talk.* Mol Cancer Res, 2004. 2: p. 523-531.

Figure 1. Number of genes changed (above random chance) by different treatments.G ene expression in MDA-MB-231 treated for 24 hours was analyzed on Affymetrix microarrays. Statistical analysis show that RA+GF causes a significant change ($p<0.05$, student's t-test) in expression of many more genes than either compound alone, while Targretin +GF was no more effective than Targretin alone. All treatments were done in triplicate. V=vehicle.



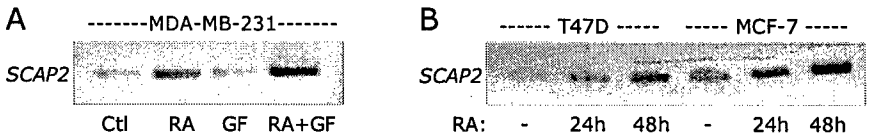


Figure 2. Induction of SCAP2/RA70 by RA+GF A) MDA-MB-231 cells were treated as in Figure 1, and SCAP2 expression was assessed by RT-PCR. B) SCAP2 is also induced by RA alone in the ER-positive and RA-sensitive cell lines MCF-7 and T47D.

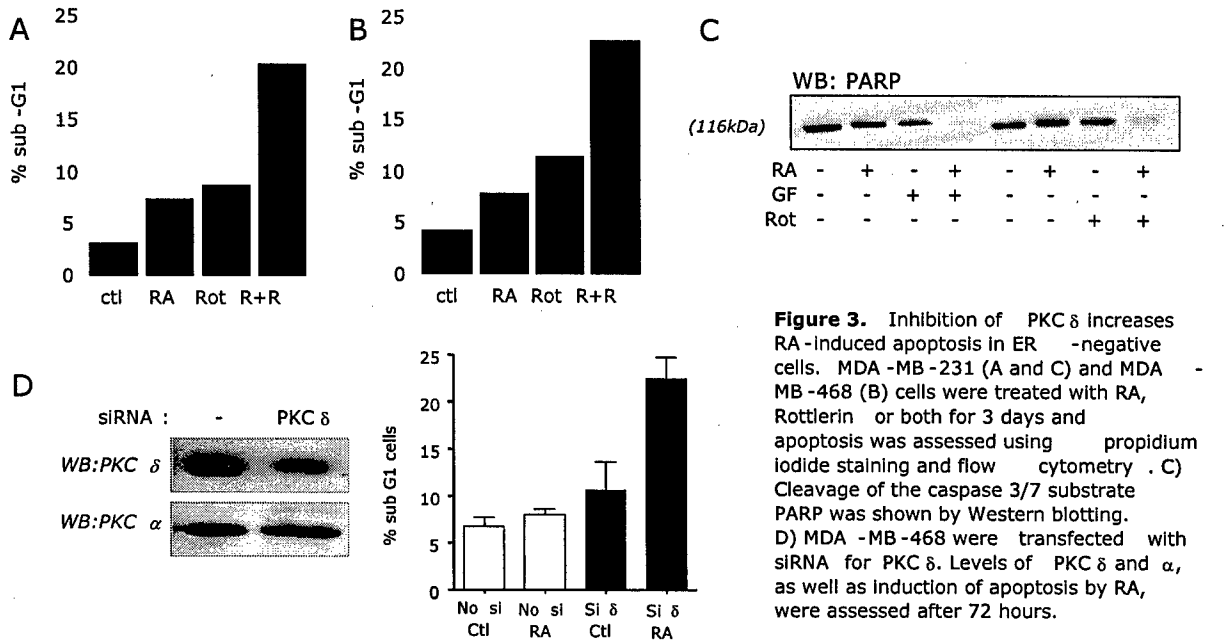


Figure 3. Inhibition of PKC δ increases RA-induced apoptosis in ER -negative cells. MDA-MB-231 (A and C) and MDA-MB-468 (B) cells were treated with RA, Rottlerin or both for 3 days and apoptosis was assessed using propidium iodide staining and flow cytometry . C) Cleavage of the caspase 3/7 substrate PARP was shown by Western blotting. D) MDA-MB-468 were transfected with siRNA for PKC δ. Levels of PKC δ and α, as well as induction of apoptosis by RA, were assessed after 72 hours.

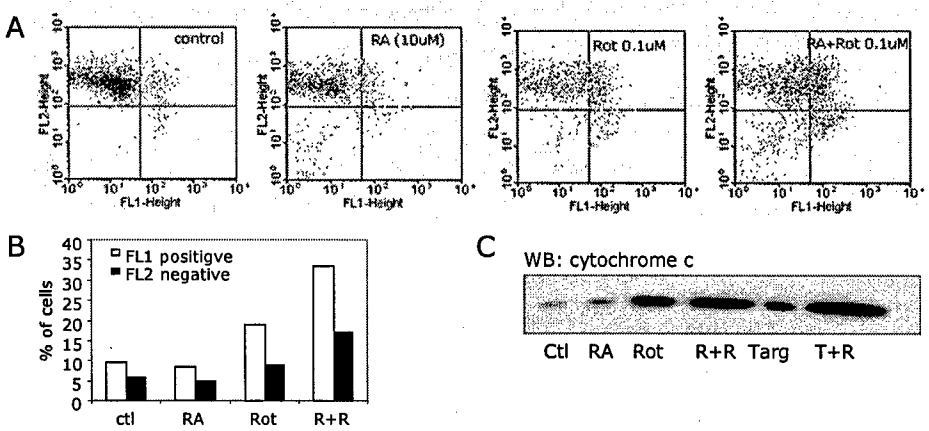


Figure 4. Loss of mitochondrial trans-membrane potential and release of cytochrome c in cells treated with RA+Rottlerin. A) $\Delta\Psi$m was measured using the fluorescent dye JC-1, which fluoresces orange (FL2 positive) in live cells with intact mitochondrial membranes, and green (FL1 positive) in apoptotic cells. B) Results from A) were analysed using CellQuest software. C) Release of cytochrome c from mitochondria was shown by Western blot analysis of S100 (cytosolic) fractions. MDA-MB-231 cells were treated as indicated for 3 days.

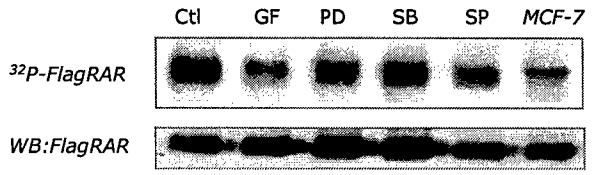


Figure 5. Inhibition of basal phosphorylation of RARα by kinase inhibitors. MDA-MB-231 cells, transiently transfected with Flag-RARα, were labeled with 32P-orthophosphate in the absence or presence of the kinase inhibitors. GF=GF109203X (PKC), PD= PD98059 (MEK), SB=SB203580, SP=SP600125 (JNK). As a comparison, the last lane shows basal phosphorylation of Flag-RARα in MCF-7 cells.

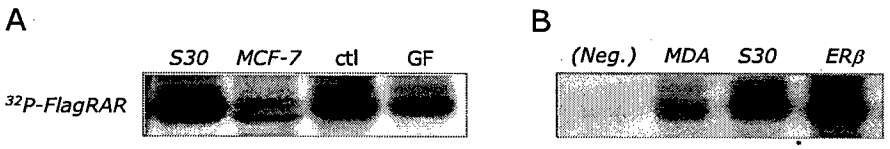


Figure 6. Increased phosphorylation of RARα in MDA-MB-231 stably expressing ERα (S30) and ERβ. *In vivo* phosphorylation of Flag-RARα was assessed as in Figure 5. The negative control (Neg.) shown in B) represents untransfected MDA-MB-231.

ERβ Sensitizes Breast Cancer Cells to Retinoic Acid: Evidence of Transcriptional Crosstalk

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Abstract

The ability of retinoids to inhibit breast cancer cell growth correlates with estrogen receptor (ER) α status, as shown by the antiproliferative effects of retinoids in ERα-positive breast cancer cells and their use as chemopreventive agents in premenopausal women. The discovery of ERβ, also present in breast cancer cells, has added a new level of complexity to this malignancy. To determine the retinoid response in ERβ-expressing breast cancer cells, we used retroviral transduction of ERβ in ER-negative MDA-MB-231 cells. Western blot and immunofluorescence confirmed expression and nuclear localization of ERβ, whereas functionality was shown using an estrogen response element–containing reporter. A significant retinoic acid (RA)–mediated growth inhibition was observed in the transduced ERβ-positive cells as shown by proliferation assays. Addition of estradiol, tamoxifen, or ICI 182,780 had no effect on cell growth and did not alter RA sensitivity. We observed that retinoids altered ERβ-mediated transcriptional activity from an estrogen response element, which was confirmed by decreased expression of the *pS2* gene, and from an activator protein response element. Conversely, the expression of ERβ altered RA receptor (RAR) β expression, resulting in greater induction of *RARβ* gene expression on RA treatment, without altered expression of *RARα*. Our data provide evidence of transcriptional crosstalk between ERβ and RAR in ERβ-positive breast cancer cells that are growth inhibited by RA. (Mol Cancer Res 2004;2(9):523–31)

Introduction

Estrogens are potent mitogens in the mammary gland that are required for normal development but are also involved in the progression of mammary carcinoma. The action of estrogen is mediated by binding to the estrogen receptor (ER), a ligand-activated transcriptional factor. Although it was initially believed that the action of estrogen was mediated by a single ER (ERα), it was subsequently determined that a second ER (ERβ) exists (1, 2). The two receptors are highly homologous in the DNA binding domain (region C) and ligand binding domain (region E). However, they greatly differ in the NH_2-terminal A/B domain and hinge region (2). The tissue distribution of ERα and ERβ is equally divergent, with ERα being most highly expressed in the pituitary, vagina, uterus, and breast and ERβ in the ovary, prostate, and lung. The presence of ERβ in the breast, albeit in lower concentration than ERα, has led to deliberation regarding its role in mammary development and tumorigenesis (3). Sixty percent to 70% of breast epithelial cells express ERβ at all stages of breast development, whereas ERα expression varies according to the developmental stage of the mammary gland (4). Studies with ERα knockout mice have shown that ERβ does not mediate E2-dependent growth and development of the mammary gland (5). Because many breast tumors express ERα alone or in combination with ERβ (6), there is interest in determining the role of ERβ in breast cancer. Some groups have found that ERβ correlates with low biological aggressiveness of breast cancer and can even inhibit proliferation and invasion of breast cancer cells (4, 7). In contrast, others have indicated that the ratio of ERα to ERβ changes during breast cancer progression, with increased expression of ERβ in relapsed patients exhibiting tamoxifen resistance (8).

The clinical approaches to controlling hormonally responsive breast cancer have primarily focused on ERα and its target genes. In patients with hormonally responsive breast cancer, current treatment involves blocking the action of ERα using anti-estrogen therapies. However, hormonal treatment is limited by the development of resistance to tamoxifen and alternative therapies targeting other signaling pathways need therefore be explored.

Retinoids are derivatives of vitamin A that induce differentiation in the treatment of acute promyelocytic leukemia and can cause growth inhibition in a variety of other cell types, including breast cancer cells (9-12). Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats (13-15). Furthermore, clinical evidence supports the benefit of retinoids for breast cancer prevention in premenopausal women (16, 17).

Retinoids mediate their effects by binding to a group of nuclear receptors [retinoic acid receptors (RAR) and retinoid X receptors (RXR)] belonging to the superfamily of nuclear receptors that includes ER. These receptors are transcription factors that heterodimerize to bind to RA response elements (RARE) present in the promoter regions of target genes (18). Interestingly, the response to retinoids in breast cancer cell lines

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seems to correlate with the expression of ERα, suggesting a possible crosstalk between the RA and the ER pathways (19, 20). We have reported previously that the expression of ERα in ERα-negative human breast cancer cell modulates RAR signaling. We found that stable expression of ERα in the ER-negative human breast cancer cell line MDA-MB-231 led to increased activity of an RARE reporter construct and sensitized the cells to growth inhibition by RA (20, 21). Because breast tumors can express both isoforms of the ER, there exists the potential for ER signaling to be mediated by ERα, ERβ, or both.

To evaluate the effects of ERβ on retinoid-mediated growth inhibition, we engineered human ERβ (hERβ) stably transduced cells from the ER-negative breast cancer cell line, MDA-MB-231, using retroviral technology. We observed several similarities between the stable ERβ-expressing cell line and the ERα-expressing breast cancer cells with regard to the growth and transcriptional response to retinoids. In addition, we noted that retinoids also inhibited the transcriptional activity from estrogen response element (ERE)–driven promoters. Our results provide evidence of transcriptional crosstalk between ERβ and RAR and support the use of retinoids to target subpopulations of breast cancer cells expressing functional ERβ.

Results

Stable Expression of ERβ

To determine if ERβ can alter retinoid-mediated growth inhibition and transcription, we generated stable expression of hERβ in the ER-negative parental MDA-MB-231 human breast cancer cell line (Fig. 1A). The ERβ cDNA was inserted upstream of an internal ribosomal entry site and the enhanced green fluorescence protein (eGFP) gene in the HC2 retroviral vector, thereby allowing us to use flow cytometric analysis to monitor transduction efficiency. MDA-MB-231 cells were transduced with either the empty HC2 retroviral vector containing only eGFP (Ctrl) or the retroviral vector expressing both ERβ and eGFP (ERβ). To achieve the highest transduction efficiency, cell sorting was done based on green fluorescence and a shift in fluorescence intensity was evident for both the retrovirus control and the ERβ-transduced cell lines (Fig. 1B). In all experiments described, both the parental cell line (MDA-MB-231) and the empty retroviral transduced cell line (Ctrl) were used as ER-negative controls. However, to avoid superfluous data, only the empty retroviral transduced cell line (Ctrl) will be shown herein. Transduced polyclonal stable cell lines were tested for their expression of ERβ by Western blots done on whole cell extracts. Protein expression of ERβ at ~55 kDa was evident in the transduced cell line (ERβ) and, as

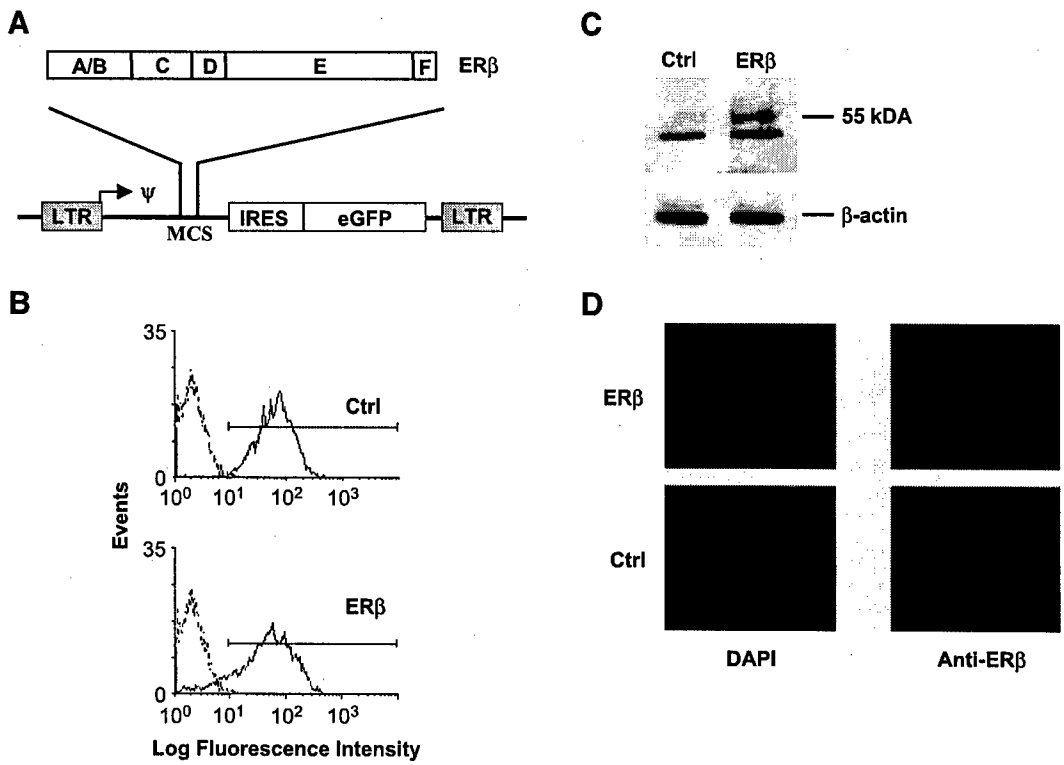

FIGURE 1. Retroviral-mediated expression of hERβ in MDA-MB-231 ERα-negative breast cancer cells. **A.** The cDNA of wild-type hERβ was cloned into the bicistronic murine stem cell virus-based retroviral vector. This vector contains a packaging signal (Ψ), multiple cloning site (*MCS*), internal ribosomal entry site (*IRES*), and *eGFP* flanked by two long terminal repeats (*LTR*). **B.** Flow cytometric analysis of transduced MDA-MB-231 cells subsequent to cell sorting. *Solid lines,* eGFP expression in the empty vector transduced cells (*Ctrl*) and ERβ-transduced cells (*ERβ*); *dashed lines,* untransduced MDA-MB-231 cells. **C.** ERβ protein expression of the transduced cells was analyzed by immunoblots using cell extracts (50 μg) and the QED anti-ERβ antibody. *β-actin,* loading control. **D.** Immunostaining of transduced cells using an ERβ antibody (*right panels*) and visualized by fluorescence microscopy. 4′,6-Diamidino-2-phenylindole staining (*DAPI*) was included to visualize the nucleus (*left panels*).

expected, was absent in the cell line expressing only the empty retroviral vector (Ctrl; Fig. 1C). Localization of ERβ to the nucleus was further confirmed using immunofluorescence (Fig. 1D).

Growth and Transcriptional Response to ERβ Ligands

Several groups have shown previously that reintroducing ERα into an ERα-negative cell line alters the proliferative response to the ERα agonist, estradiol (22, 23). In the ERβ stably transduced cell line (ERβ), treatment with estradiol, tamoxifen, or ICI 182,780 resulted in no significant change in proliferation (Fig. 2A). This agrees with a previous study in which the growth rate of ERβ-positive breast cancer cells was unaffected by estradiol or ICI 182,780 (4). To validate the functionality of the expressed ERβ, we investigated the transcriptional activity from a reporter construct containing three tandem EREs in the presence of known ERβ ligands. We observed an induction of transcription in response to estradiol and complete inhibition of transcription from this reporter in the presence of tamoxifen and ICI 182,780 in the ERβ-positive cells. As expected, these ligands had no effect in the cell line expressing only the retroviral vector (Ctrl). Increased baseline transcription in the ERβ cells may be attributed to residual estrogen in medium or to ligand-independent receptor activity. These data confirm the functionality of ERβ in the stably transduced cell line.

Growth Response of the ERβ Stable Transfectant to RA

We compared the growth inhibitory effect of RA in ERα-positive (ERα) and ERβ-positive cells (ERβ). The ERα stable cells are an ERα-positive subclone (S30) of MDA-MB-231 and were not derived from retroviral transduction (22). However, both cell lines were derived from the identical parental cell line, MDA-MB-231. After 6 days of treatment with RA, we observed significant growth inhibition only in the cells expressing ERα or ERβ (Fig. 3A). To determine if the growth inhibitory action of RA is altered by ERβ ligands, we compared the effect of RA in the presence of the ERβ agonist (E2) and antagonist (ICI 182,780). As seen in Fig. 3B, 6 days of treatment with RA alone resulted in ~50% growth reduction for ERβ-expressing breast cancer cells, and this remained unchanged regardless of the presence of ER agonistic or antagonistic ligand.

RA Alters ERβ-Mediated Gene Expression

The expression of ERα-regulated genes can be altered by RA (24, 25). To determine if similar transcriptional effects would be observed in ERβ-positive cells, we studied the expression of *pS2*, a known ER-responsive gene containing an ERE in its promoter region. Expression of ERβ increases basal expression of *pS2* in absence of ligand as compared with the ER-negative Ctrl. Although *pS2* is induced by stable transduction of ERβ alone, treatment with estradiol increases the expression of this gene. In ERβ-positive cells, induction of *pS2* by estradiol is inhibited by 24 hours treatment with RA (Fig. 4A). Densitometry analysis was done on two independent experiments to determine the pS2/glyceraldehyde-3-phosphate dehydrogenase ratio. E2 treatment enhanced pS2 expression

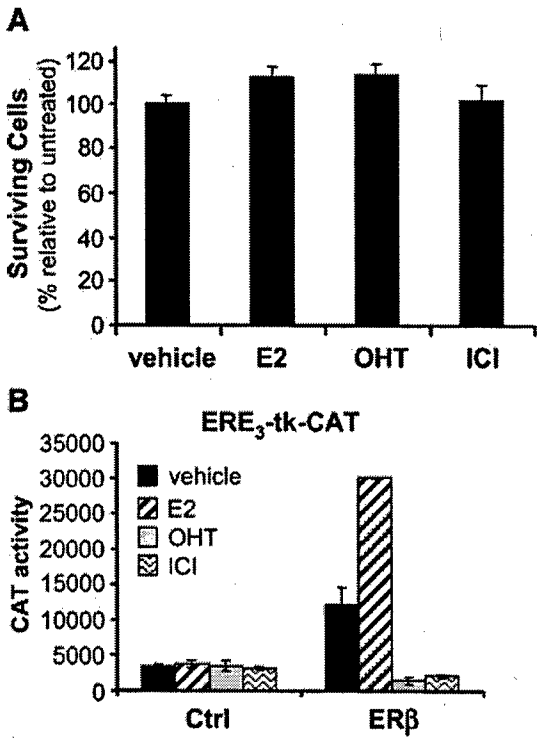

FIGURE 2. Effect of ERβ ligands on the growth and transcriptional properties of ERβ-transduced cells. **A.** After 6 days of treatment with 10^{-7} mol/L estradiol (*E2*), 10^{-7} mol/L tamoxifen (*OHT*), or 10^{-7} mol/L ICI 182,780 (*ICI*), cell number was assessed using sulforhodamine B staining as described in Materials and Methods. *Columns,* average of two separate experiments in quadruplicate. **B.** ERβ-transduced cells exhibit ligand-dependent transcriptional activity from an ERE_3-tk-CAT reporter. *Columns,* average of two independent experiments in triplicate, normalized with βGAL.

2-fold, whereas treatment with RA alone or with E2 decreased *pS2* expression by 50% of the untreated cells. In addition, on a synthetic promoter containing three tandem EREs, RA inhibits transcription, and this inhibition is maintained even in the presence of estradiol (Fig. 4B). These data indicate that ER-mediated transcription is inhibited by RA and that an ER agonist cannot rescue the transcriptional inhibitory effect of RA.

RA Decreases Induced Activator Protein Activity in ERβ-Positive Breast Cancer Cells

The growth inhibitory effect of retinoids in breast cancer cells has often been attributed to its inhibitory action on activator protein (AP-1)–mediated transcription (26, 27). In contrast, ERα can increase AP-1 activity in response to estradiol. Using a reporter construct from the collagenase promoter containing an AP-1 response element [Coll(−73)Luc], we studied the effect of ERβ ligands and RA on AP-1 activity using treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to confirm the functionality of the reporter. Unexpectedly, we observed an increase in AP-1 activity on E2 treatment in the ERβ stable transfectants, whereas both 4-hydroxytamoxifen (OHT) and ICI 182,780 treatment did not alter AP-1 transcription (Fig. 5). These results were unanticipated because, unlike ERα, E2 has been characterized previously as an inhibitor of ERβ-mediated

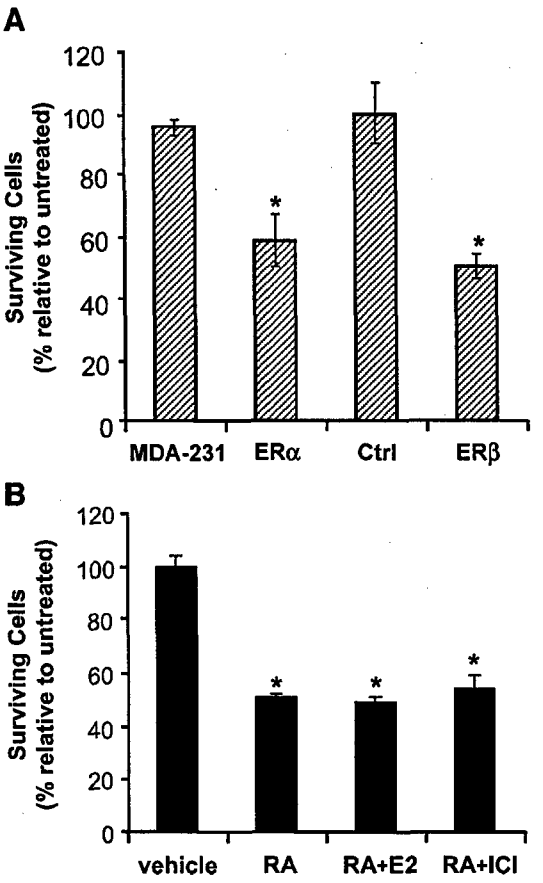

FIGURE 3. ERβ-transduced cells are growth inhibited by RA. **A.** Viable MDA-MB-231 cells stably expressing either the empty retroviral vector (*Ctrl*), *ERα*, or *ERβ* were assessed using sulforhodamine B staining as described in Materials and Methods after 6 days of 10^{-5} mol/L RA treatment and compared with untreated cells. **B.** RA-mediated growth inhibition in ERβ-transduced cells is unaltered by estradiol (10^{-7} mol/L, *E2*) or ICI 182,870 (10^{-7} mol/L, *ICI*) after 6 days. *Columns*, average of at least three different experiments in triplicate. *, $P < 0.05$, statistically significant from the parental cell line (**A**) or treatment with vehicle (**B**), calculated using Dunnett's test.

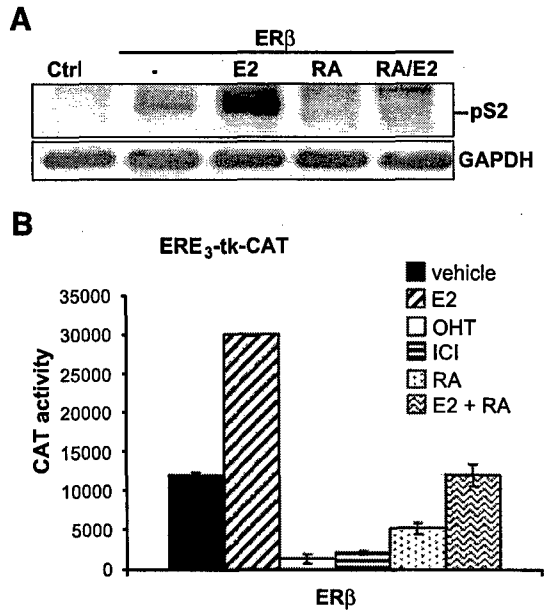

FIGURE 4. Analysis of gene regulation in ERβ-transduced breast cancer cells. **A.** Northern blot analysis of pS2 expression in empty retroviral vector (*Ctrl*) or ERβ-transduced cells (*ERβ*). Cells were treated with 10^{-5} mol/L RA, 10^{-7} mol/L estradiol (*E2*), or a combination of both ligands for 24 hours. Total cellular RNA (20 μg) was loaded in each lane as well as the corresponding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). **B.** RA inhibits transcription from an ERE. Cells were cotransfected with ERE_3-tk-CAT and CMV-βGAL and treated with the indicated ligands for 48 hours.

AP-1 activity and OHT as an activator (28). The Ctrl cell line was unaffected by ER ligands or RA (data not shown). When compared with vehicle-treated cells, treatment with RA alone did not significantly alter AP-1-mediated transcription. However, when ERβ-expressing cells are cotreated with RA and E2, the AP-1 stimulatory action of estradiol, as well as that of TPA, is blocked by RA.

ERβ Alters the Expression of RARβ2

To determine if there was reciprocal crosstalk between ERβ and RAR, we also observed the effect of ERβ on RAR-mediated transcription. Some groups have shown that an increased level of RARα correlates with RA-mediated growth inhibition (29, 30), whereas others attribute the inhibition of growth by RA to induction of RARβ2 (31). To determine the level of these nuclear receptors in our stably transfected cell lines, we assessed the expression of *RARα* and *RARβ2* by Northern blot and semiquantitative reverse transcription-PCR, respectively (Fig. 6). We noted no significant change in expression level of *RARα* in the Ctrl cell line, the ERα-positive or ERβ-positive stable transfectants, before or after RA treatment (Fig. 6A). Because the induction of *RARβ2* has been associated with response to retinoids in certain types of cancer, we also assessed the expression of this gene in our system by semiquantitative reverse transcription-PCR. It is well documented that *RARβ2* is present at much lower levels than *RARα* in breast

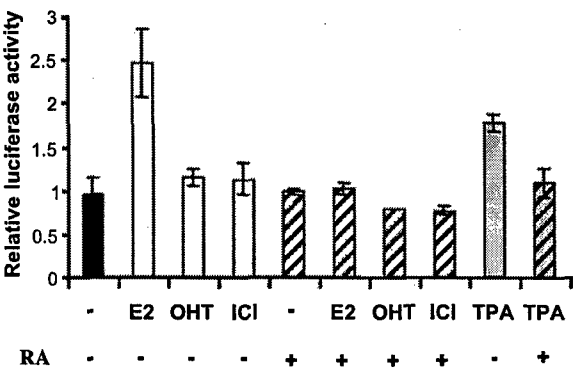

FIGURE 5. Analysis of AP-1-mediated transcriptional activity in ERβ-transduced breast cancer cells. ERβ-transduced cells were transfected with 1 μg Coll(−73)Luc and 1 μg CMV-βGAL expression vector and treated with vehicle, 10^{-7} mol/L estradiol (*E2*), 10^{-7} mol/L tamoxifen (*OHT*), 10^{-7} mol/L ICI 182,780 (*ICI*), 100 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*), or a combination of each ligand with 10^{-6} mol/L RA for 24 hours. *Columns*, mean transcriptional activity of at least two independent experiments in triplicate; *bars*, SEM.

cancer cells, rendering it difficult to perform a simple Northern or Western blot. In contrast to *RARα*, we observed significant differences in the level of expression of *RARβ2*. Expression of *β-actin*, the linearity of which was verified by titration, shows that the samples contained equivalent amounts of RNA. In the ERα or ERβ stable transfectants, expression of *RARβ2* was suppressed in the absence of RA and, unlike the ER-negative cells, *RARβ2* expression levels were strongly induced in response to RA (Fig. 6B).

Effect of ERβ on the Transcriptional Activity of a Transiently Expressed RARE

To further characterize the effect of ERβ on *RARβ2* expression, we transiently expressed a reporter driven by the RARE of the RARβ2 promoter in the stably transduced cell lines and assessed transcriptional activity in the absence and presence of 10^{-6} mol/L all-*trans* retinoic acid (Fig. 7). We observed that cells expressing the ERβ receptor, as compared with the ER-negative cells, displayed significantly lower basal activity from this promoter in the absence of RA (Fig. 7B). Transient transfection of only the tk-CAT part of the reporter did not differ between the cell lines, indicating that the effect of ERα or ERβ on transcriptional activity was due to the βRARE and not any part of the thymidine kinase promoter (data not shown). We detected an ~10-fold decrease in basal activity from the βRE-tk-CAT in the presence of ERα or ERβ (Fig. 7B). On addition of RA, there was a strong induction of transcriptional activity in the cells that had a suppressed baseline and a weaker induction in the parental cells (Fig. 7A). This transcriptional effect was only observed on an RARE, because reporter constructs containing vitamin D response element, thyroid hormone response element, or peroxisome proliferator response element were unaffected by the expression of ERα or ERβ (data not shown). Thus, expression of ERα or ERβ results in a greater induction of *RARβ2* expression when compared with ER-negative cells.

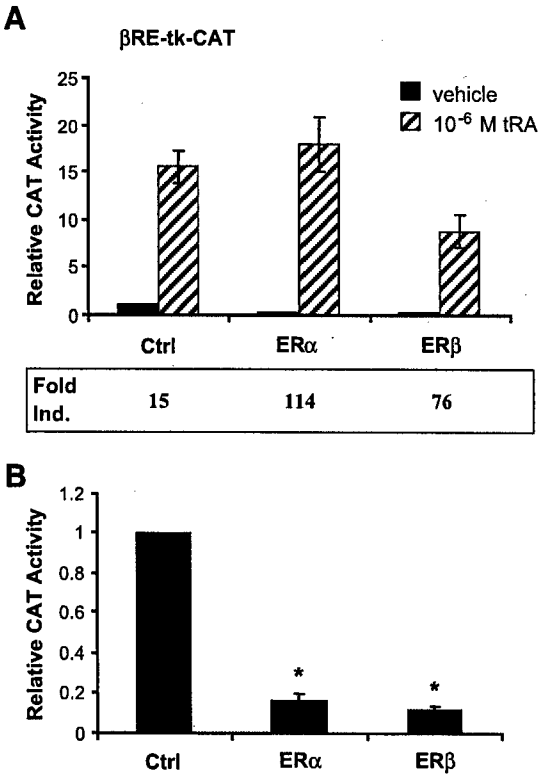

FIGURE 7. Expression of ERα or ERβ increases the fold induction of transcriptional activity from the βRARE promoter by suppressing basal activity in the absence of RA. **A.** Retinoid-induced transcriptional activity in the absence (*Ctrl*) or presence of ERα or ERβ. Cells were transfected with 1 μg βRE-tk-CAT and 1 μg CMV-βGAL expression vector and treated with 10^{-6} mol/L RA or vehicle for 48 hours. *Box*, fold induction (*Fold Ind.*) for each cell line. *Columns*, mean transcriptional activity of at least three independent experiments in triplicate; *bars*, SEM. **B.** Transcriptional activity from the βRE-tk-CAT in absence of ligand. *Columns*, chloramphenicol acetyltransferase (*CAT*) activity relative to the ER-negative cell line (*Ctrl*). *, $P < 0.05$, statistically significant from the *Ctrl*, calculated using Dunnett's test.

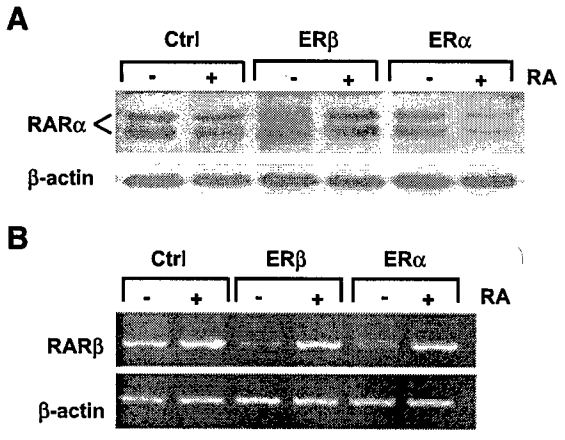

FIGURE 6. Retinoid receptor expression and regulation by RA in ER-positive cells. **A.** RARα Northern blot analysis of total cellular RNA (20 μg) isolated after 24 hours of 10^{-5} mol/L RA (or vehicle) treatment. *β-actin*, loading control. **B.** Basal RARβ2 expression is suppressed but inducible by RA in ER-expressing cells. Reverse transcription-PCR analysis of RARβ2 in cells treated with RA (10^{-6} mol/L) or vehicle for 24 hours using *β-actin* to control for variability in cDNA.

An Active ERβ Is Required to Maintain RARβ2 Inducibility

The ligands OHT and ICI 182,780 both inhibit the transcriptional activity of ERβ. We tested the activity of the βRARE reporter in the presence of these antagonists and the agonistic ligand estradiol in the ERβ-positive cells (Fig. 8A). As expected, OHT, ICI 182,780, and estradiol had no effect on the Ctrl empty retroviral transfected cell line. In cells stably expressing ERβ, OHT and ICI 182,780 completely released inhibition of the RAR-mediated transcription and restored baseline activity to the same level as seen in the ER-negative cell line (Ctrl). Addition of estradiol, which activates ERβ via the AF-2 domain, further decreases βRARE activity in these cells. Because it is known that these ligands can alter the expression levels of ERα, we verified their effects on the expression of the stably transduced ERβ cells after 24 hours of treatment. Although there were no changes in mRNA expression (data not shown), the protein levels were significantly altered in the presence of the various ligands (Fig. 8B). As reported previously with ERα, estradiol and ICI 182,780 both down-regulate expression of ERβ. Although the expression of

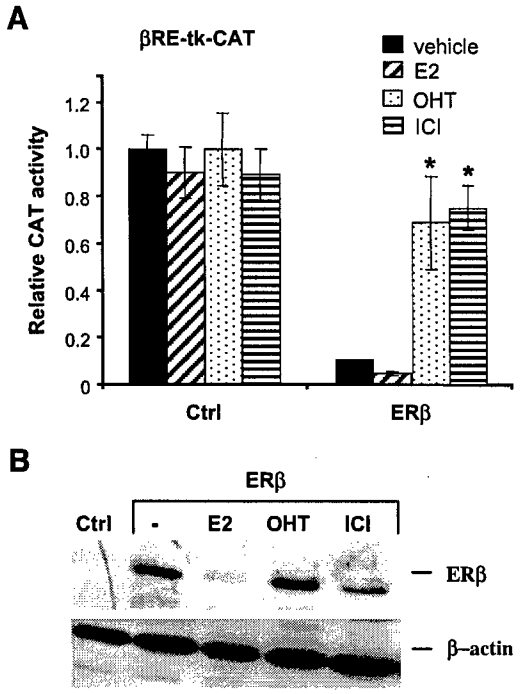

FIGURE 8. ERβ ligands alter transcriptional activity from the βRE-tk-CAT. **A.** Baseline transcriptional activity of the βRE-tk-CAT was evaluated after treating the cells 1 hour post-transfection with vehicle, 10^{-7} mol/L tamoxifen (*OHT*), 10^{-7} mol/L ICI 182,780 (*ICI*), or 10^{-7} mol/L estradiol (*E2*). (*E2*). *Columns*, chloramphenicol acetyltransferase (*CAT*) activity relative to the ER-negative cell line (*Ctrl*). *, $P < 0.05$, statistically significant differences between the vehicle and the treated samples. **B.** Immunoblot of whole cell extracts (50 μg) depicting the effect of the ERβ ligands mentioned above on ERβ protein expression after 24 hours. Cells were also immunoblotted for *β-actin* as a loading control.

ERβ is similarly down-regulated by both of these ligands, only the antagonist ICI 182,870 increases the transcriptional activity from the βRARE. These data indicate that inhibition of ERβ transcriptional activity allows transcription from the βRARE. Conversely, activation of ERβ transcription with estradiol inhibits βRARE-mediated transcription.

Discussion

Breast cancer is a hormone-dependent malignancy, with standard therapy directed at regulating ER-mediated signaling. In premalignant and malignant breast lesions, ER expression is significantly increased (32). The discovery of the ERβ in both normal and malignant breast tissues has added a new level of complexity, although the expression of ERβ in the breast is less abundant than ERα and there is controversy regarding its role in breast physiology and tumorigenesis (33).

Retinoids have shown some therapeutic potential for the treatment of breast cancer (16, 17). Although they do not target ER directly, there is considerable evidence correlating the presence of ERα with RA sensitivity (10, 20, 34-36). Importantly, clinical trials using a retinoid derivative have shown efficacy in preventing contralateral breast cancer in premenopausal women exclusively, further suggesting a role for ERα in RA-mediated growth inhibition (37). Because ERβ can also be detected in breast cancer cells, we wished to determine its prognostic implications in the management of breast cancer with retinoids. For this purpose, we engineered ERβ-positive breast cancer cells using retroviral transfection of the ERα-negative MDA-MB-231 cell line. Although several ERβ isoforms have been identified in human breast cancer tissue and cell lines, we used the wild-type hERβ of 530 amino acids (6).

Expression of ERβ was confirmed by Western blot and immunofluorescence. Functionality of the transduced ERβ was shown by transient transfection, using a synthetic ERE-containing reporter construct, and by estradiol-mediated activation of the *pS2* gene. Although stable expression of ERβ restored ligand-dependent transcription, the growth properties of the stable cell lines were unaffected by the ERβ ligands estradiol, tamoxifen, or ICI 182,780. These results agree with those reported previously in which ER ligands did not alter the proliferation of ERβ-expressing stable cell lines (4, 38). These data suggest that the estradiol-driven neoplastic process of the breast that has been described for ERα may not be pertinent to ERβ. Although ER ligands did not alter the proliferation of ERβ-transduced cells, we observed that retinoids inhibited proliferation of ERβ-positive and ERα-positive breast cancer cells and that cell growth was inhibited regardless of cotreatment with ER ligands.

The mechanism for retinoid-mediated growth inhibition is not well understood. However, there is evidence that retinoids suppress estradiol-mediated proliferation and transcriptional activity and can antagonize the proliferative effects of AP-1 (27, 39). In the ERβ-transduced cells, we show that RA can repress ERE-mediated transcription and decreases estradiol-activated endogenous gene expression (*pS2*). However, repression of ERβ activity alone cannot explain the growth inhibitory properties of retinoids because ER ligands do not alter the proliferation of ERβ-positive breast cancer cells.

Increased AP-1 activity generally leads to activation of cell proliferation signals (40, 41). Because the growth inhibitory mechanisms of retinoids have, in part, been attributed to the antagonism of this activity, we explored the possibility that ERβ cells may have altered AP-1 activity in response to RA. Several groups have shown that, unlike ERα, anti-estrogens activate ERβ-mediated AP-1 activity, whereas E2 is antagonistic (28, 42). In contrast, using a reporter construct from the collagenase promoter containing an AP-1 response element [Coll(−73)Luc], we noted that E2 increased AP-1 activity in these cells. Despite the activation of AP-1 activity, the proliferation rate of ERβ-positive cells was unaffected by treatment with E2. Furthermore, the anti-estrogens OHT and ICI 182,780 did not alter AP-1-mediated transcription in our stably transduced ERβ cells. These results, which contradict those observed in transient transfections of ERβ, are in accordance with those reported in another ERβ stably transfected MDA-MB-231 cell line in which anti-estrogens did not activate AP-1 response elements (38). We also report that treatment with RA did not significantly alter basal AP-1-mediated transcription but decreased E2-induced and TPA–induced AP-1 activity. Although RA antagonism of AP-1 activity is not specific for E2 induction of this reporter, we have nevertheless shown that RA can alter ERβ-mediated transcription from both ERE-driven and AP-1-driven promoters in breast cancer cells stably expressing ERβ.

Because transcriptional crosstalk has been reported between ERα and RAR, we also examined the effect of ERβ expression on RA-mediated pathways. The growth inhibitory action of retinoids has often been attributed to increased expression of RARα and to RARβ2 induction (43-45). We found that the expression of *RARα* was unchanged in response to RA but that the basal expression of *RARβ2* RNA, as determined by reverse transcription-PCR (Fig. 6B) and RNase protection assay (data not shown), was significantly lower in cells expressing ERα or ERβ than in parental ER-negative cells. Induction of *RARβ2* expression has been associated with retinoid response in a variety of cancer cell types and provides another example of the crosstalk between ER-mediated and RAR-mediated pathways in human breast cancer cells (44-46). Using ER ligands, we determined that the function of ERβ in altering RAR-mediated transcription takes precedence over its expression levels. Although both ICI 182,780 and E2 decrease ERβ protein expression, these ligands oppose each other in their action on the RARβ promoter. We have shown previously that the NH_2-terminal region of ERα, including the DNA binding domain, was important for mediating transcriptional crosstalk with RAR. Although ERβ varies greatly from ERα in the NH_2-terminal region, there are some similarities. For example, it has been reported that both receptors can bind p300 at the NH_2 terminal in absence of ligand (47). Therefore, it remains a possibility that ERβ or ERα interaction with RAR transcription pathway may involve squelching for limited known or unknown cofactors. Stable cell lines using ERβ deletion mutants or ERβ variants will provide greater insight into this transcriptional interaction. In addition, it may be of interest to study the effect of ERβcx expression on retinoid activity in breast cancer cells. This isoform has been detected in human breast cancer, shows preferential dimerization with ERα, and has a dominant negative effect on ligand-dependent ERα reporter gene transactivation (48).

In conclusion, we provide evidence of nuclear receptor signaling crosstalk between ERβ and RAR in human breast cancer cells. Given the promiscuity of coactivators and corepressors with different nuclear receptors, it is not surprising that crosstalk exists between the different nuclear receptor families. We show that RA can significantly decrease the growth of ERβ-positive breast cancer cells in the presence or absence of ER ligands, thereby supporting the use of retinoids for the management of ERβ-positive breast cancer.

Materials and Methods

Cells

MDA-MB-231 (clone 10A) and the ERα-positive subclone (S30) were obtained courtesy of Dr. V.C. Jordan (Northwestern University Medical School, Chicago, IL). All cell lines were routinely cultured in α-MEM phenol red–free medium (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 5% charcoal-stripped serum. For the culture of S30 cells, 0.5 μg/mL G418 (Life Technologies) was added to the above medium. All cells were maintained in 5% CO_2 at 37°C in a humidified atmosphere.

Construction of Stable Cell Lines

The hERβ expression vector (1,590 bp) was kindly provided by Dr. S. Mader (Département de Biochimie, Université de Montréal, Montreal, Quebec, Canada). Retroviral vectors were constructed by cloning the above cDNA in the multiple cloning site of the murine stem cell virus retroviral vector (HC2). This technique has been described previously in detail (21). Concentrated virus was used to infect MDA-MB-231 cells. Pooled populations of transduced cells were routinely analyzed by flow cytometry for eGFP expression, thus confirming expression of the bicistronic RNA and the stable expression of ERβ.

Western Blots

Whole cell extracts were isolated from confluent 150 mm plates of transduced cells as described previously (21). Whole cell extracts were also isolated from cells treated with 10^{-7} mol/L tamoxifen (kindly provided by Dr. A.E. Wakeling, Zeneca, Macclesfield, United Kingdom), 10^{-7} mol/L ICI 182,780 (Sigma Chemical Co., St. Louis, MO), and 10^{-7} mol/L estradiol (Sigma Chemical) and treated for 24 hours. Protein lysates (50 μg) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were probed using the ERβ antibody (QED Biosciences, San Diego, CA) and incubated with secondary antibody at a 4,000-fold dilution prior to analysis by chemiluminescence. Membranes were exposed to anti-β-actin antibody (Sigma Chemical) to control for loading.

Northern Blots

Expression of RARα and pS2 were analyzed by Northern blot as described previously (10). Briefly, total RNA (20 μg) was isolated 24 hours post-treatment with indicated ligands and electrophoresed on a 1% formaldehyde/agarose gel and blotted onto nitrocellulose filter. The filters were hybridized to radiolabeled probes of RARα (*Pst*I fragment) or pS2, washed, and autoradiographed.

Immunofluorescence

Cells were grown on coverslips until semiconfluent monolayers were obtained and fixed with 4% cold paraformaldehyde in PBS. Coverslips were washed with PBS-0.5% Triton X-100 containing 10% FCS (Life Technologies) for 5 minutes at room temperature. Incubation with 1:50 anti-ERβ (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-0.1% Triton X-100 was done for 3 hours in a humid chamber. Cells were washed extensively with PBS-0.01% Triton X-100 and staining was done using Alexa Fluor 546–conjugated anti-goat secondary antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in PBS-0.01% Triton X-100 for 30 minutes. Cells were washed again and 2 μg/mL 4′,6-diamidino-2-phenylindole (Molecular Probes) solution was added for 5 minutes to visualize nuclei. Coverslips were mounted onto glass slides using Immuno-Mount (Shandon, Inc., Pittsburgh, PA) and cells were visualized with an Olympus BX51 fluorescence microscope (Olympus, Melville, NY). An oil immersion (100×) objective was selected for the observations.

Cell Proliferation Studies

Cells were seeded in 24-well plates at a density of 2,000 cells per well. In the treated cells, a final concentration of 10^{-5} mol/L all-*trans* retinoic acid, 10^{-7} mol/L E2, 10^{-7} mol/L OHT, or 10^{-7} mol/L ICI 182,780 was replenished 1 day after seeding and subsequently on days 3 and 5. Controls for treated cells contained identical concentrations of vehicle alone. After 6 days of treatment, cells were fixed in 10% trichloroacetic acid and subsequently stained with sulforhodamine B (Sigma Chemical). Sulforhodamine B is an aminoxanthene dye that binds to basic amino acid residues and gives an index of culture cell protein that is linear with cell number (49). Bound sulforhodamine B was solubilized in 10 mmol/L unbuffered Tris and absorbance was measured at 570 nm in a microplate reader for quadruplicate samples.

Analysis of RARβ Expression

Expression of RARβ was assessed by reverse transcription-PCR as described previously (21). Briefly, cDNA was prepared from 1 μg RNA and PCR amplification was done using RARβ2 primers. To ensure the validity of the β-actin PCR as a loading control, the β-actin samples were titrated and we confirmed that the PCR result was within linear range.

Transient Transfections and Chloramphenicol Acetyltransferase Assays

Cells were plated at 2×10^5 cells per well in six-well plates and allowed to adhere overnight in phenol red–free α-MEM medium supplemented with 5% charcoal-stripped fetal bovine serum. Transfections were done using FuGENE (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's guidelines. Transfections of the βRE-tk-CAT and ERE_3-tk-CAT have been described previously (21). Briefly, reporter plasmid (1 μg) was transfected with pCMV-βGAL plasmid (1 μg) as an internal control using a ratio of 2:1 FuGENE (Boehringer Mannheim) to DNA. Cells were treated with the indicated ligands after 5 hours and harvested 48 hours post-transfection. For transfection of an AP-1 response element, the above methodology was followed with the Coll(−73)Luc reporter, except that cells were treated with the indicated ligands for 24 hours. Luciferase assay was done in accordance with the manufacturer's guidelines (Promega, Madison, WI) and measured using a Lumat LB-9507 luminometer (Perkin-Elmer Instruments, Darmstadt, Germany). 12-*O*-tetradecanoylphorbol-13-acetate (100 ng/mL) was used as a positive control.

Statistical Analysis

Results from representative experiments are shown as means of the number of replicates. Statistical analysis was done using Dunnett's test, wherein statistical significance was noted for $P < 0.05$.

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